Institut für Veterinärpathologie
der Vetsuisse-Fakultät
Universität Zürich

Direktor Prof. Dr. med. vet. Andreas Pospischil

Arbeit unter der Leitung von Dr. med. vet. Nicole Borel

Evidence for *Parachlamydia* in bovine abortions

Inaugural-Dissertation

zur Erlangung der Doktorwürde der
Vetsuisse-Fakultät Universität Zürich

vorgelegt von

Silke Anita Ruhl

Tierärztin
von Heidelberg, Deutschland

genehmigt auf Antrag von

Prof. Dr. med. vet. Andreas Pospischil, Referent
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Index

Tables

Figures

Evidence for *Parachlamydia* in bovine abortion

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1. Abstract

Bovine abortion of unknown infectious aetiology still remains a major economic problem. In this study, we focused on new possible abortigenic agents such as *Parachlamydia acanthamoebae* and *Waddlia chondrophila*. Retrospective samples (n = 235) taken from late-term abortions in cattle were investigated by real-time diagnostic PCR for *Chlamydiaceae, Parachlamydia* spp. and *Waddlia* spp., respectively. Histological sections of cases positive by real-time PCR for any *Chlamydia*-related agent were further examined by immunohistochemistry using specific antibodies.

Chlamydophila abortus was detected only in three cases (1.3%) by real-time PCR and ArrayTube Microarray playing a less important role in bovine abortion compared to the situation in small ruminants in Switzerland. By real-time PCR as many as 43 of 235 (18.3%) cases turned out to be positive for *Parachlamydia*. The presence of *Parachlamydia* within placental lesions was confirmed in 35 cases (81.4%) by immunohistochemistry. The main histopathological feature in parachlamydial abortion was purulent to necrotizing placentitis (25/43). All 235 cases were negative for *Waddlia* by real-time PCR.

Parachlamydia should be considered as a new abortigenic agent in Swiss cattle. Since *Parachlamydia* may be involved in lower respiratory tract infections in humans, bovine abortion material should be handled with care given the possible zoonotic risk.

Keywords: abortion; cattle; *Parachlamydia*; *Waddlia*; real-time PCR; immunohistochemistry

2. Introduction

Abortion in bovines is of worldwide economic importance. In Switzerland, 14,000 to 28,000 bovine abortion cases are reported every year resulting in a loss of 22 to 45 million Swiss Francs per year (Hässig et al., 2000). While *Neospora caninum* and Bovine Viral Diarrhea Virus (BVDV) have been shown to be important abortigenic agents, most cases of abortion cases (67.7%) remain of unknown aetiology (Reitt et al., 2007).

In cattle, several disease syndromes due to *Chlamydia* infection have been reported (Shewen, 1980; Idtse, 1984; Perez-Martinez and Storz, 1985; Storz and Kaltenboeck, 1993). *Chlamydophila (Cp.) abortus* and *Cp. pecorum* (Longbottom, 2004) are known to infect cattle and cause abortion and other urogenital tract infections, pneumonia and conjunctivitis. Bovine abortion due to *Cp. abortus* occurs during the sixth to eighth months of gestation particularly among heifers in their first pregnancy. Among experimentally induced abortions, placentitis is the most consistent and striking pathological feature (Idtse, 1984; Perez-Martinez and Storz, 1985).

In 2001, the first case of bovine chlamydial abortion was reported in the canton of Graubunden, Switzerland (Pospischil et al., 2002b). In this region, *Cp. abortus* is endemic in small ruminants (Borel et al., 2004). Moreover, *Cp. abortus* is known to cause zoonotic infection in humans which, in the case of pregnant women, can result in spontaneous abortion (Pospischil et al., 2002a; Longbottom and Coulter, 2003). *Cp. pecorum* has also been associated with abortion and conjunctivitis in ruminants (Aitken et al., 1990) but the zoonotic potential of *Cp. pecorum* is unknown (Longbottom, 2004). *Waddlia chondrophila*, another member of the *Chlamydiales*, is a newly recognised abortigenic agent in bovines (Rurangirwa et al., 1999; Dilbeck-

Robertson et al., 2003). This obligate intracellular bacterium was isolated from an aborted fetus in the United States (Dilbeck et al., 1990) and in Germany (Henning et al., 2002). Further a serological study supported the abortigenic role of *Waddlia* in bovine species (Dilbeck-Robertson et al., 2003). Recently, the zoonotic potential of *W. chondrophila* was suggested by an association of anti-*Waddlia* antibodies and sustained contact with animals (Baud et al., 2007). In that work, *Waddlia* seroprevalence was significantly higher in women who had had sporadic and recurrent miscarriages than in control women ($p<0.001$) (Baud et al., 2007).

Other *Chlamydia*-related organisms, such as *Parachlamydia acanthamoebae* might also be involved in bovine abortion (Borel et al., 2007b).
Thus, among 235 bovine placental specimens, 43 were positive for *Chlamydia*-related organisms by 16S rRNA PCR, of which 70% (30/43) were confirmed by immunohistochemistry performed using anti-*Parachlamydia* antibodies (Borel et al., 2007b).

Given this first evidence of a role for *Parachlamydia* in bovine abortion (Borel et al., 2007b), we further studied these 235 bovine placental samples, using a recently developed TaqMan® real-time PCR for *Parachlamydia* and *Waddlia*, and performed immunohistochemistry on positive samples using specific polyclonal mouse antibodies directed against the corresponding *Chlamydia*-related organism to demonstrate the agent directly in the placenta.

3. Material and Methods

3.1. Abortion cases

Placental tissue (1 - 4 cotyledons per case) of 235 randomly selected late-term bovine abortions were collected during the breeding seasons 2003/2004 and submitted to the Cantonal Laboratory of Veterinary Bacteriology, Chur, Switzerland.

Subsequently, placental samples were both fixed in 4% formalin and embedded in paraffin wax and frozen at -20°C.

3.2. DNA extraction and real-time PCR

DNA was extracted using the AquaPure Genomic Tissue Kit (BioRad, Rheinach, Switzerland) according to the manufacturer's instructions. Overnight proteinase K treatment was performed and DNA was resuspended in 100 μl of DNA hydration solution provided in the kit.

All cases were examined by real-time PCR for the presence of DNA of *Chlamydiacaeae*, *Parachlamydia* and *Waddlia*.

3.2.1. Chlamydiaceae PCR

The real-time PCR was conducted on an ABI 7500 using a modified version of the procedure of Everett et al. (1999). Primers Ch23S-F (5'-CTGAAACCAGTAGCTTATAAGCGGT-3'), Ch23S-R (5'-ACCTCGCCGTTTAACTTAACTCC-3'), and probe Ch23S-p (FAM-CTCATCA TGCAAAAGGCACGCCG-TAMRA) were selected.

It was used to amplify a 111-bp product specific for members of the family *Chlamydiaceae*. 2.5 μl of extracted DNA was added to a commercial Master Mix (TaqMan® Fast Universal PCR Master Mix, Applied Biosystems, Foster City, CA, USA) with final concentration of 5 pmol/μl of each primer and the probe (Microsynth, Balgach, Switzerland) and 12.50 μl of 2x buffer to yield a final volume of 25 μl. The amplification was performed with initial denaturation (95°C, 10 min), followed by 45 cycles of denaturation at 94°C for 15 s, 60°C for 60 s (Ehricht et al., 2006). Cycle threshold (Ct value) of < 38.00 was considered as positive.

3.2.2. *Parachlamydia* and *Waddlia* PCR

Amplification and PCR product detection of *Parachlamydia* were performed with the ABI Prism 7000 sequence Detection system (Applied Biosystems), using recently described primers (PacF + PacR) and probe (PacS) (Casson et al., 2008). Cycling conditions were 2 min at 50°C, 10 min at 95°C, followed by 45 cycles of 15 s at 95°C and 1 min at 60°C (Casson et al., 2008).

For *Waddlia* detection, Primer WadF and WadR as well as probe WadS were used (Goy and Greub, 2008).

Samples were considered negative if no amplification was observed during all 45 cycles. The mean Ct values were compared using the unpaired Student t-test.

3.3. ArrayTube (AT) Microarray for species identification of *Chlamydiaceae*

The design and print pattern of the microarray version were as described (Borel et al., 2007a). Briefly, the chip carried 28 probes for species identification (four probes each for *C. trachomatis*, *Cp. pneumoniae* and *Cp. psittaci*, three probes for *C. muridarum*, *Cp. pecorum*, *Cp. caviae* and *C. suis*, two probes for each of *Cp. abortus* and *Cp. felis*). The chip also carried three genus-specific probes (two for *Chlamydophila* and one for *Chlamydia*), five probes for the closest relatives, i.e. *Simkania negevensis* and *Waddlia chondrophila*, as well as four positive controls (consensus probes), and one internal staining control (biotin marker). The AT assay was performed as described elsewhere (Borel et al., 2007a).

3.4. Histology

Haematoxylin and eosin (HE) stained histological sections of all placenta specimens (n = 235) were examined for the type and degree of placentitis and/or vasculitis.

3.5. Immunohistochemistry

3.5.1. *Chlamydiaceae*

Paraffin wax sections were investigated for the presence of chlamydial antigen by using a *Chlamydiaceae* family– specific mouse monoclonal antibody directed against the chlamydial lipopolysaccharide (LPS, Clone ACI-P, Progen, Heidelberg, Germany). Detection was performed with a detection kit (Dako ChemMate, Dako, Glostrup, Denmark) according to the manufacturer's instructions. Briefly, sections were dewaxed in xylene and rehydrated through graded ethanol to water. Antigen retrieval was performed by 10 min enzyme digestion (Pronase, Dako). To inhibit the endogenous peroxidase activity, the slides were immersed in peroxidase-blocking solution for 5 min at room temperature (RT) and incubated with the primary antibody diluted in antibody diluent for 60 min at RT. The sections were incubated for 30 min at RT with the link-antibody, developed in 3-amino, 9-ethyl-carbazole (AEC) substrate solution for 10 min at RT, and counterstained with haematoxylin. By using the antibody diluent instead of the primary antibody a negative control of each section was performed. Intestinal tissue from gnotobiotic piglets experimentally infected with porcine *Chlamydia suis* strain S45 was used as a positive control (Guscetti et al., 2000).

3.5.2. *Parachlamydia*

Histological sections of cases positive for *Parachlamydia* by real-time PCR were investigated for the presence of *Parachlamydia* using a specific mouse polyclonal antibody produced as described elsewhere (Casson et al., 2007). Optimisation experiments for immunohistochemical protocols are described elsewhere (Borel et al., 2007b). To test the placental specimens, we used the mouse

polyclonal antibody directed against *Parachlamydia* at dilution of 1:1,000. Detection was performed with the detection Kit (Dako) according to the manufacturer's instructions. Antigen retrieval was performed by repeated microwave heating (750W for 10 min) in citrate buffer (pH 6,0, Target Retrieval Solution, Dako ChemMate). By immersing the slides in peroxidase-blocking solution for 5 min at RT endogenous peroxidase activity was blocked. Primary antibody was incubated for 1h. Incubation with the link-antibody and substrate solution and counterstaining with haematoxylin was preformed as described above. Negative and positive controls of each section were included as described previously (Borel et al., 2007b).

3.5.3. *Waddlia*

Histological sections of cases positive for *Waddlia* (if any) and *Parachlamydia* by real-time PCR were investigated with the same immunohistochemical protocol as described in 2.5.2. The primary mouse antibody directed against *Waddlia* was used at dilution of 1:2,000 (60 min at RT).

4. Results

4.1. Abortion cases

As already reported earlier (Borel et al., 2007b), purulent and/or necrotizing placentitis was observed in 149 of 235 cases (63.4%). Placentitis along with vasculitis was present in 26 of 235 cases (11.1%). Examination of placental smears for *Coxiella burnetii* and sera from the dams for *Brucella abortus* and IBR-IPV are described elsewhere (all cases were negative for these respective agents, Borel et al., 2006).

4.2. *Chlamydiaceae*

Three of the 235 cases (1.3%) were positive for *Chlamydiaceae* by real-time PCR. The three positive samples were identified as *Cp. abortus* using the ArrayTube Microarray.

All three *Cp. abortus* positive cases showed typical histopathological lesions of a chlamydial abortion, such as purulent and/or necrotizing placentitis and vasculitis. Immunohistochemistry for *Chlamydiaceae* in these three positive cases was positive in two cases and doubtful in one case (Table 1).

4.3. *Parachlamydia*

43 out of the 235 cases (18.3%) were positive with the *Parachlamydia*-specific real-time PCR. Among them, only nine had already been identified by the 16SigF-16SigR broad-range PCR we used in our previous work (Borel et al. 2007b) to detect the presence of any *Chlamydiales*. Overall, 25 of the 43 positive cases (58.1%) showed a purulent and or necrotizing placentitis and three (7.0%) an additional vasculitis (Figure 1). Eight (18.6%) cases showed no alteration and 10 (23.2%) were autolytic. By immunohistochemistry (IHC), 35 of the 43 cases positive by PCR (81.4%) were confirmed positive with the antibody elicited against *Parachlamydia* (Table 2, Figure 2). Conversely, each of the 43 PCR positive placental samples was negative with the antibody directed against *Waddlia*.

Interestingly, the mean Ct value of samples with a positive IHC result for *Parachlamydia* was significantly lower than that of samples with a negative IHC result ($p=0.011$, see Figure 3). Moreover, among the 34 cases positive by real-time PCR and negative with the broad-range 16Sif-16SigR PCR (which has a sensitivity about 100x lower when compared to the new real-time PCR), 28 (82.3%) were confirmed by immunohistochemistry. This is in contrast to the only two (6.8%) out of 29 cases

with a negative real-time PCR and with a broad-range PCR identifying a *Chlamydiales* exhibiting less than 95% homology with *Parachlamydia acanthamoebae.*

4.4. *Waddlia*

All 235 cases were negative for *Waddlia* by real-time PCR.

5. Discussion

Although purulent and/or necrotizing placentitis in bovine abortion is often observed (Borel et al., 2006), most of these cases remain of unknown infectious aetiology. We therefore focused on new emerging abortigenic agents including *Chlamydiaceae* and two *Chlamydia*-related organisms: *Waddlia* and *Parachlamydia*. We selected placental specimens of late term abortions since *chlamydiae* have been shown experimentally to multiply primarily in the cotyledons, causing severe inflammation and necrosis leading to abortion (Idtse, 1984; Perez-Martinez and Storz, 1985).

Cp. abortus and *Cp. psittaci* do not seem to play an important role in bovine abortion in Switzerland since only three samples out of 235 (1.8%) were positive by real-time PCR for *Chlamydiaceae*. All three were classified as *Cp. abortus* using the ArrayTube Microarray method. This confirms the results of a previous study (Borel et al., 2006). In contrast, a large number of bovine late-term cases of abortion (43/235) were found to be positive for *Parachlamydia* by real-time PCR. As mentioned in the results section, only 21% of them had previously been identified using the 16Sif-16SigR broad-range PCR, demonstrating that the *Parachlamydia*-specific real-time PCR is more sensitive than the conventional broad-range PCR, that had been

developed for taxonomic purposes rather than for diagnostics. More importantly, the positive real-time PCR results were unlikely to be false positives since most (81%) of the 34 positive cases, which were negative with the 16Sif-16SigR broad-range PCR were confirmed by immunohistochemistry. Conversely, the fact that only 6.9% of cases with a negative *Parachlamydia* PCR were found to be positive by immunohistochemistry performed with home - made polyclonal anti-*Parachlamydia* antibodies, confirmed the specificity of this immunohistochemistry. Finally, the strong correlation between the DNA copies (i.e. Ct values) obtained by using the real-time PCR and the presence of positive immunohistochemistry further validate the results of the real-time PCR

Overall, these findings confirm the results of a previous study in which *Parachlamydia* was detected in bovine abortion cases by 16S rRNA PCR (Borel et al., 2007b). All 43 cases positive for *Parachlamydia* real-time PCR were negative for all other abortigenic agents investigated in a previous study (Borel et al., 2006). This further supports the role of *Parachlamydia* in bovine abortion, and since it was the exclusively identified abortigenic organism, it allows us to define more precisely the histopathological characteristics of parachlamydial abortion. Purulent to necrotizing placentitis was mainly seen but concurrent vasculitis in the placenta was only present in three (7.0%) cases positive for *Parachlamydia*. Thus the combination of placentitis and vasculitis does not seem to be a typical pathomorphological feature of parachlamydial abortion in the way that it is in *Cp. abortus*-associated abortion. Inflammation and necrosis of the placenta were present in almost 60% of the *Parachlamydia*-positive cases. In chlamydial abortion, severe placentitis will result in placental insufficiency and abortion. A similar mechanism could trigger parachlamydial abortion. However, corresponding fetal tissues of the investigated

cases were not available, and possible lesions and antigen distribution in the fetus require further investigation.

IHC was performed on the 43 real-time positive cases, 35 of them (81.4%) were positive for *Parachlamydia*. The amount and distribution of parachlamydial antigen within the bovine placenta estimated by IHC was in general comparable to the situation in ovine enzootic abortion caused by *Cp. abortus* (data not shown). Abundant parachlamydial positive granular elements were mainly intracytoplasmic within trophoblastic epithelium correlating with the growth of *Parachlamydia* within mammalian cell lines (Corsaro and Greub, 2006). This is the first study showing the presence of *Parachlamydia* within trophoblastic epithelial cells. However, further experiments should be carried out to investigate the significance of these findings. Positive labelling of parachlamydial antigen was similarly present as in cases of ovine enzootic abortion (Longbottom and Coulter, 2003), and more frequent than described in chlamydial abortion in cattle (Borel et al., 2006). Negative IHC results in eight cases could be explained by lower sensitivity of the IHC compared to the real-time PCR. Indeed, Ct values were in five out of these eight cases higher than 41 (41.02-44.88), suggesting a low amount of parachlamydial genome copy numbers in the sample, likely explaining the negative IHC results. For the other three cases with Ct values ranging from 37.1 to 39.5, the negative IHC results may have been due to sampling different sites from the fresh placenta for real-time PCR and IHC.

Suprisingly, *Waddlia* was not detected in any of the 235 cases of bovine abortion. This finding was in contrast to our expectations since *Waddlia* has been described in bovine abortion in Germany (Henning et al., 2002) and in USA (Dilbeck et al., 1990). In our study *Waddlia* was not detected by either the sensitive and specific real-time PCR or IHC in the cases positive by real-time PCR for *Parachlamydia*. The absence of cross-reactivity between these two agents was

expected given the results of a previous investigation that showed little cross-reaction between members of different *Chlamydiales* families (Casson et al., 2007). *Waddlia* has been cultivated from the fetal heart in the case of bovine abortion from Germany (Henning et al., 2002) and from fetal lung and liver in the case in the USA (Dilbeck et al., 1990). In our study we examined only placental specimens as fetal organs were not available. It is, however, unknown if *Waddlia* colonizes the placenta. Further studies on the significance of *Waddlia* in ruminant abortion should include the examination of both, the placenta and the fetal organs.

As demonstrated in this study, bovine abortion due to placentitis of unknown aetiology may be caused by novel *chlamydiae*, which have remained unrecognized when using routine diagnostic methods such as specific PCR for *Chlamydiaceae* and immunohistochemistry with anti-*Chlamydiaceae* antibodies. In this study we applied recently developed tools such as real-time PCR and IHC for *Parachlamydia* and *Waddlia*. These new methods were used on field samples and proved to be sensitive and specific for the detection of *Parachlamydia*. *Parachlamydia* was not only detected by PCR but also with species-specific immunohistochemical protocols, demonstrating the agent within the placental lesions (see above). The applied protocols are suitable for routine diagnostics and should be used to complement the current examination procedures on cases of bovine abortion. The advantage of species-specific real-time PCR methods is its higher sensitivity compared to a broad-range PCR, likely due to use of lasers and fluorescent probes to detect the PCR products and to the much smaller amplicon targeted. The length of the amplicon is especially important in determining PCR sensitivity on formalin-fixed samples, given the fragmentation of the DNA during preparation of histological samples. Another advantage of the real-time PCR developed by Casson et al. (2008) and by Goy and Greub (2008) is their possible use for large epidemiological studies, since these PCR

are compatible with the 384-well format. However, with such specific PCRs, new or closely related *Chlamydia*-related organisms are not detected. Broad-range PCR methods targeting the 16S gene or other conserved regions of the chlamydial genome are more suitable for this purpose and have been applied in our previous study (Borel et al., 2007b) to screen for the range of *Chlamydiales* occuring in bovine abortion.

Having this first evidence of *Parachlamydia* as a potential new abortigenic agent in cattle, its isolation from cases of bovine abortion is essential to definitively prove its importance. Our study has revealed evidence of *Parachlamydia* in bovine abortion, another potential zoonotic agent that may cause bronchitis and pneumonia in humans (Greub and Raoult, 2002; Greub et al., 2003a, 2003b). Thus the general zoonotic risk of handling aborted bovine material should be emphasized since there are already several known zoonotic agents, such as *Coxiella burnetii*, *Brucella abortus* and *Chlamydophila abortus* causing severe disease in humans.

6. Conclusion

Whereas *Waddlia* seems not play an important role in bovine abortion in Switzerland, our evidence suggests that *Parachlamydia* should be considered as a new abortigenic agent in cattle. The main histopathological feature in parachlamydial abortion was purulent to necrotizing placentitis possibly resulting in placental insufficiency. New sensitive methods such as real-time PCR and IHC specific for *Parachlamydia* are suitable for routine diagnostics.

Since *Parachlamydia* may be involved in lower respiratory tract infections in humans, a potential zoonotic risk arising from bovine abortion material should be taken in consideration.

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Table 1

Results for *Chlamydiaceae* screen by LPS IHC, real-time PCR and ArrayTube Microarray

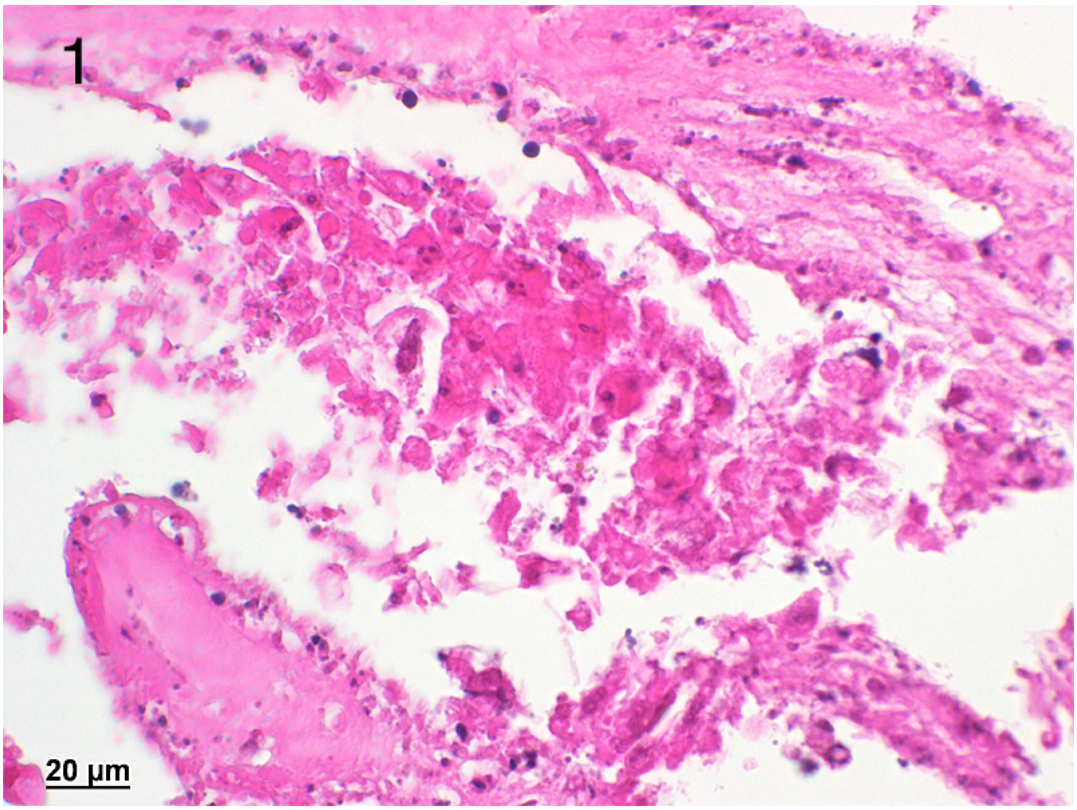
Case No.	Placentitis	Vasculitis	IHC	real-time PCR	ArrayTube Microarray	Ct-Values (mean values)
1	purulent/necrotizing	yes	quest	+	*Cp. abortus*	36.45
2	necrotizing	yes	+	+	*Cp. abortus*	23.10
3	purulent/necrotizing	yes	+	+	*Cp. abortus*	18.26

Table 2

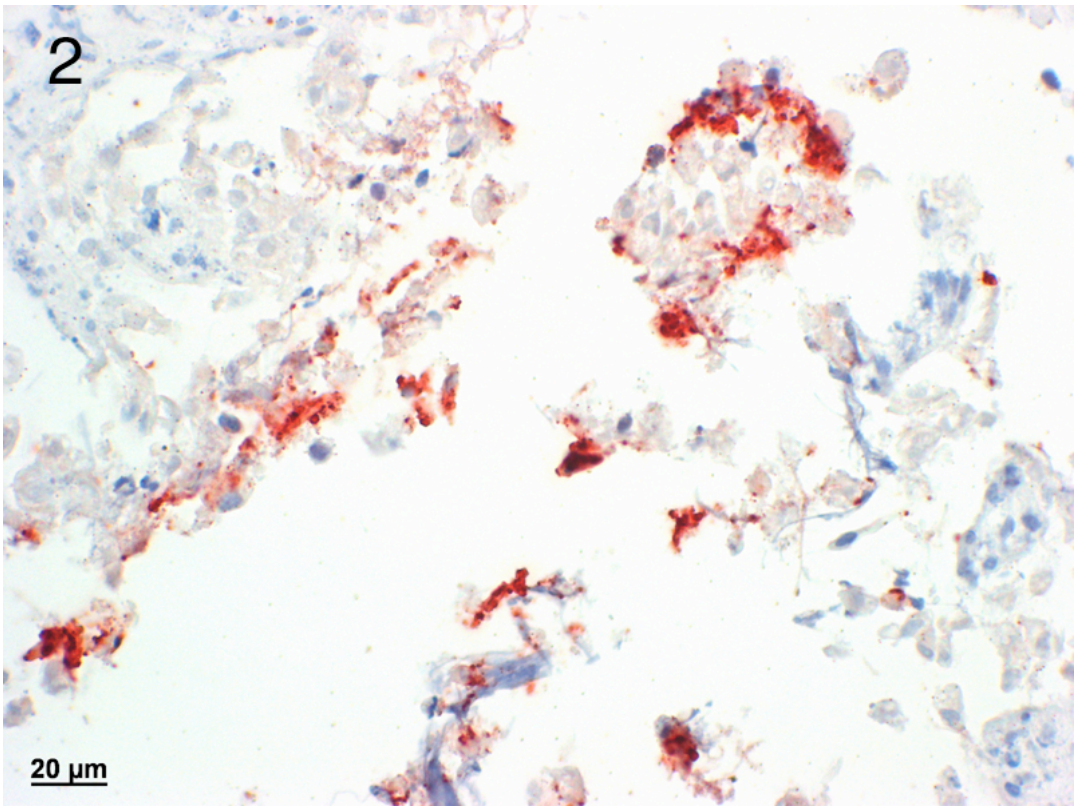
Results for *Parachlamydia* screen by parachlamydial IHC and real-time PCR for *Parachlamydia*

Case No.	Placentitis	Vasculitis	IHC	real-time PCR	Ct-Values (mean values)
4-6	purulent/necrotizing	yes	+	+	39.66, 40.59, 43.13
7-16	purulent/necrotizing	no	+	+	33.15, 35.23, 37.29, 37.37, 37.43, 38.19, 38.37, 39.02, 42.46, 43.89
17-21	purulent/necrotizing	no	-	+	39.47, 39.48, 41.02, 41.27, 43.67
22-28	necrotizing	no	+	+	37.41, 37.60, 37.73, 38.24, 38.73, 39.95, 39.96
29-37	autolysis	no	+	+	37.68, 38.11, 38.34, 38.57, 38.84, 39.15, 39.72, 39.95, 41.38

38	autolysis	no	-	+	37.08
39-44	none	no	+	+	35.45, 38.27, 38.32, 39.37, 40.10, 42.07
45, 46	none	no	-	+	42.61, 44.88

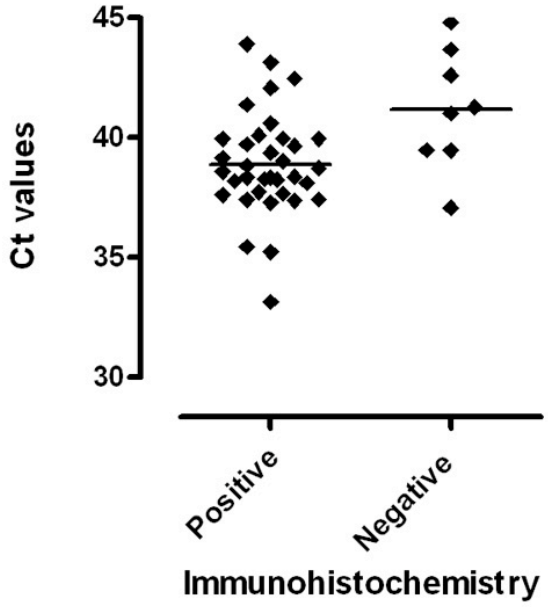


Placenta; cow, case no. 9. Histopathology of a case positive for *Parachlamydia* by real-time PCR (Ct value 37.29) and immunohistochemistry showing purulent to necrotizing placentitis. Haematoxylin and eosin staining.



Placenta; cow, case no. 9. Immunohistochemistry with the anti- *Parachlamydia* Antibody. Presence of positive granular reaction within trophoblastic epithelium. AEC/peroxidase method, haematoxylin counterstain.

3



Correlation of mean Ct value of samples with a positive immunohistochemistry (IHC) Result for Parachlamydia and of samples with an negative IHC result (p=0.011)

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10. Curriculum Vitae

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2000 – 2006	Studium der Veterinärmedizin an der Justus von Liebig Universität Giessen, Deutschland
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